MOLECULAR CHARACTERIZATION OF AN ALTERED MATING BEHAVIOR IN DROSOPHILA MELANOGASTER VESTIGIAL MUTANTS

A Thesis
by
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ABSTRACT

MOLECULAR CHARACTERIZATION OF AN ALTERED MATING BEHAVIOR IN DROSOPHILA MELANOGASTER VESTIGIAL MUTANTS

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The mating behavior of the fruit fly, *Drosophila melanogaster,* consists of a male courtship ritual used to entice females to mate and females accepting or rejecting copulation with males. A stock of *D. melanogaster* with the *vestigial* wing mutation (*vg*) has been found to display a different male courtship behavior and, despite having a wing deficiency that prevents a major part of the courtship ritual, the males do not have a reduced courtship rate with females of their same stock. Receptivity to *vg* males is a genetic trait that can be artificially selected for in *vg* females. Using choice tests and brother-sister pair matings, recombinant inbred (RI) lines of *D. melanogaster* were created that varied in the percentage of females that would mate with *vg* males over wild type males (30-100%). These lines were used in molecular genetic tests to attempt to determine which genes are responsible for the differences in female behaviors. The first approach that was used was amplified fragment length polymorphism (AFLP) analysis. While there were differences in AFLPs among different lines, there were no correlations between banding patterns and female behavior. All

attempts to sequence AFLP bands that were different in order to determine which genes they represented were unsuccessful. The next approach used was to sequence a candidate gene and to compare the sequences of this gene from each RI line. *Delta* was chosen to sequence because of its neurogenic properties and its implication in sexual isolation between two other *Drosophila* species. Sequencing of the *Delta* exons was successful in all lines, but no sequence differences were found that correlated with the differences in female behavior. Based on this information, this thesis serves to evaluate the effectiveness of AFLP analysis in detecting genetic differences associated with mating behaviors.

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INTRODUCTION

Charles Darwin defined sexual selection as "a struggle between the males for possession of the females" that results in more or less offspring being produced (Darwin, 1871). In nature, both sexes develop strategies to maximize their contribution of genes to the next generation. These strategies can be based on intrasexual or intersexual selection. Intrasexual selection involves two members of the same sex competing for access to mates, while intersexual selection involves preferences for one individual of the opposite sex over another based on certain trait(s) (Darwin, 1871). New techniques in genetic analysis have opened up the ability to search for genes responsible for both types of sexual selection. Furthermore, the study of sexual selection is an ideal mechanism for the study of Darwinian evolution.

Drosophila as a Model Organism

Drosophila melanogaster is a suitable species to study Darwinian evolution because it has a short generation time and females produce large numbers of offspring, which makes it relatively simple to track changes over time. It is particularly well suited to the study of sexual selection because its mating behavior and genetics have been so extensively studied. Due to the abundance of molecular markers and visible mutations, identifying candidate genes associated with sexual selection is achievable. Having the entire *D. melanogaster* genome sequence further facilitates this goal (Adams et al., 2000).

Mating Behavior in Drosophila

In most species, there is a difference in mate selection between the sexes; females tend to be particular about which males they will mate with, while males tend to mate with any receptive female. This gender contrast stems from the difference in the investment that each sex contributes to their offspring (Trivers, 1972). The largest disparity is in the gametes that each sex produces. Males have many smaller, motile, relatively inexpensive gametes, whereas females make few larger, sessile, relatively expensive gametes. Because of this anisogamy, males and females are limited in their reproductive success by different factors (Bateman, 1948; Parker, Baker, & Smith, 1972). Males have nearly infinite gametes and are only limited by the number of females that they can locate and with which they can successfully mate. Females are limited by the number of gametes they can produce in a lifetime; therefore, every female needs to ensure that each of these gametes is fertilized by a male that will either help her survival or reproductive success, or her offspring's survival or reproductive success (Trivers, 1972). Gowaty, Steinichen, & Anderson (2003) calculated the sperm to egg size ratio for *D. melanogaster* to be 0.08 (female gamete size > male gamete size) and reported only 500 progeny produced from 4,600 sperm transferred to females, which supports the ideas presented by Trivers (1972). They also found that males tend towards a continuous sexual interest in females, while females are generally "repulsed" by males, and avoid reproduction until a suitable mate is identified (Gowaty, Steinichen, & Anderson, 2003).

Female choice is often based on secondary sexual characteristics of a male, such as coloring, fancy plumage, or courtship displays. Variations in these characteristics can often communicate a male's fitness level, an indirect benefit to a female such as more attractive

sons or offspring with better genes, or their ability to provide direct benefits to a female such as shelter, food, or protection (Talyn & Dowse, 2004). Females may also respond to auditory cues, namely courtship songs (Chang & Kang, 2002), nuptial gifts (Alcock, 2005), or visual cues such as body size or courtship dance (Blanckenhorn et al., 2000; Frantsevich & Gorb, 2006). In terms of natural selection, a female can optimize her fitness by choosing the best male available (Borgia, 1979). In *Drosophila,* males don't provide any resources to the female, but by choosing the best mate a female can increase the viability of her offspring. Females who choose a mate based on courtship signals from males tend to produce more viable offspring (Talyn & Dowse, 2004).

The mating ritual of *D. melanogaster* may only last a few minutes, but it involves multiple sensory cues exchanged between males and females. Generally speaking, male flies must locate and decide to court females, while females decide whether or not to mate with a male that is courting her (Dickson, 2008). Males are innately able to discriminate between males and females and can learn to discriminate between receptive and unreceptive females (Dickson, 2008). They make these discriminations based on cuticular hydrocarbons. These hydrocarbons act as pheromones and can be volatile compounds that are detected with olfactory receptors on the antennae or maxillary palps, such as *cis*-vaccenyl acetate, which suppresses male-male courtship, or non-volatile compounds that are detected by contact through gustatory receptors on the proboscis and forelegs, such as 7-pentacosene, which may increase the chance of a male continuing to court a female (Dickson, 2008; Ferveur, 2005; Greenspan & Ferveur, 2000). The females most likely to be receptive are sexually mature virgins and a male will indiscriminately court any female he perceives to be receptive (Bastock & Manning, 1955; Dickson, 2008). The first step in the courtship ritual is for the

male to approach a female and tap it with his foreleg. In doing so, he senses the pheromones on the female and can decide to continue courting if this is an appropriate object to court. During the approach and tapping, the female fly is most likely still mobile, either walking or flying intermittently. Sensing the male's pheromones will cause her to become more receptive and slow her movement (Greenspan & Ferveur, 2000). The male can then begin his courtship song, which is produced through wing vibrations and acts as auditory stimulation for the female (Bastock & Manning, 1955; Dickson, 2008; Greenspan & Ferveur, 2000). As he "sings", he may move back and forth around the female, stimulating her visually (Bastock & Manning, 1955; Greenspan & Ferveur, 2000). After making the song and approaching the posterior of the female, the male will try to lick her genitalia and then attempt copulation (Bastock & Manning, 1955). At any time while the male is courting her, a female can express her unwillingness to mate by walking or flying away, fluttering her wings, kicking at the male, twisting her abdomen away from the male, or fully extruding her ovipositor (Bastock & Manning, 1955; Dickson, 2008; Lasbleiz, Ferveur, & Everaerts, 2006). Conversely, when a female is receptive to copulation, she will cease walking, spread her vaginal plates, and may partially extrude her ovipositor (Bastock & Manning, 1955; Dickson, 2008; Lasbleiz, Ferveur, & Everaerts, 2006). A male will not attempt to mount and copulate with a female unless he detects that she has spread her vaginal plates when he licks her genetalia (Bastock & Manning, 1955). If the female signals that she is unreceptive, the male can either stop courting or repeat the ritual. Females usually reject males at least once before allowing them to copulate, which allows the female sufficient time to assess whether the male is of the same species (Bastock & Manning, 1955).

Females base their receptivity on their sexual maturity, mating status, and the relative attractiveness of the male. Females will not mate within the first 5-8 hours of their adult life and reach sexual maturity one to two days after eclosing. If a female is generally receptive to mating, she decides on a mate largely based on the quality of his courtship song. Part of the courtship song is species specific and is used by the female for species recognition (Kyriacou & Hall, 1982; Ritchie, Halsey, & Gleason, 1999). Female fruit flies also show a preference for males of their own species that have more "energetic" courtship songs, which tend to be larger males (Talyn & Dowse, 2004). Larger males produce more offspring and have higher longevity than smaller males, so females may be choosing males that can provide genetic benefits to their offspring (Partridge & Farquhar, 1983; Partridge, Hoffmann, & Jones, 1987). Additionally, females mate faster and more frequently with males that have higher amounts of the cuticular hydrocarbon 7-tricosene (Grillet, Dartevelle, & Ferveur, 2006). It is thought that levels of this pheromone may also indicate higher genetic quality in males (Grillet, Dartevelle, & Ferveur, 2006).

Wing Mutations in Drosophila

Because the courtship song is integral to a male fly's mating success, stocks with wing mutations that potentially inhibit a male's ability to produce a song should show reduced success with wild type females. Males homozygous for *apterous* mutations, which cause the wings to be deformed or absent, have a hugely reduced mating rate with wild type females compared to wild type males with wild type females (0.8 % vs. 57%), although *apterous* mutants also show reduced non-wing courtship as well (Ringo, Werczberger, & Segal, 1992). The *raised* mutation, which causes a raised wing phenotype, prevents males

from making the courtship song, but does not affect non-wing courtship ability. Males with this mutation also show a significantly reduced mating rate with wild type females compared to wild type males with wild type females (3% vs. 71%) (McRobert, Schnee, & Tompkins, 1995).

Previous work in our laboratory has focused on *D. melanogaster* with the vestigial-winged mutation. The *vestigial* (*vg*) gene is located on chromosome 2R and codes for a protein (Vg) that is 453 amino acids long. The protein product is integral to wing and haltere development; without Vg, wings and halteres will not be made (Williams, Bell, & Carroll, 1991; Williams, Paddock, & Carroll, 1993). Vg also has the role of specifying future indirect flight muscle cells and directing their differentiation into muscle cells (Anant, Roy, & VijayRaghavan, 1998; Sudarsan et al., 2001).

The *vg* locus has nearly 400 induced or spontaneous mutant alleles that range in phenotype from homozygous lethality, to complete loss of wing and haltere structures, to no visible phenotype. The classic mutant allele, vg^{1} *or* vg^{BG}, has the phenotype of reduced wings and halteres and was first described in 1911 by Thomas Morgan. This mutation is known to have a 412 element, an 8,000 base retrotransposon, inserted in the third intron of the *vg* gene (Williams, Atkin, & Bell, 1990; Williams & Bell, 1988). This insertion causes a truncated mRNA transcript to be made that ends in the 5' long terminal repeat of the 412 element, excluding approximately 800 base pairs (Zider et al., 1996). The mutation causes apoptosis to occur in cells in the wing pouch in the early third instar, a lack of cell proliferation in the wing disc and, consequently, a reduction in mitosis to occur in cells in the late third instar (Bownes & Roberts, 1981; Fristrom, 1969; O'Brochta & Bryant, 1983).

Flies with the *vg* mutation show altered courtship behavior. Males cannot produce a normal courtship song because of the nature of their mutation but they also exhibit a non-wing courtship element that differs from wild type males. *vg* males initially approach females from the side, whereas Oregon-R wild type (*wt*) flies initially approach females from behind (Stockdale, 2004). Because females with this mutation cannot base their mating decision on the courtship song, they base their preference among *vg* males on a visual cue, as they choose fewer *vg* males compared to wild type males in the dark (Aldridge, 2005). Aldridge (2005) showed that females from *vg* laboratory stocks exhibit a mixed preference, mating with *vg* males over *wt* males roughly 50% of the time. The preference for *vg* males can be artificially selected to create true-breeding lines of flies in which *vg* females always mate with *vg* males over *wt* males, thus this particular female choice behavior has a genetic basis (Aldridge, 2005).

Genes and Behavior

Identifying genes that control or influence behaviors can be difficult for a number of reasons. A behavior is defined as "the way in which a person or animal acts in response to a particular situation or stimulus" ("Behavior," New Oxford American Dictionary) and has many influences that occur at different times. Behaviors are caused by inputs to neural networks in the brain that then signal some sort of response, usually muscle movement. The neurons involved in producing a behavior are set up during development, so gene activity does not have to occur at the same time as the behavior or in the same anatomical location that the behavior happens. Additionally, many behaviors are in response to external stimuli, which further confuses the origins of a behavior (Greenspan & Ferveur, 2000). In terms of

genes that control behaviors causing reproductive isolation between populations, known as "speciation genes" (Coyne, 1992), it is extremely difficult to study these genes between populations that no longer inter-breed. It is better to examine populations that appear to be in the incipient stages of speciation because they have not yet developed hybrid inviability or hybrid sterility (Orr, 2005).

In *Drosophila,* pre-zygotic isolation tends to arise between two species faster than post-zygotic isolation (Coyne & Orr, 1989). Populations in the very early stages of speciation should show polymorphisms in the genes that could cause prezygotic isolation, specifically, genes that control male and female sexual behavior (Wu et al., 1995). Because of the difficulties associated with locating behavior genes, only a handful of genes known to cause behavioral isolation have been identified in any animal species thus far. Several isolating behaviors between various *Drosophila* species have been mapped to chromosome regions (some more specific than others), but few genes have been definitively identified as contributing to behavioral isolation. *Drosophila ananassae* and *Drosophila pallidosa* are species that are sexually isolated by female discrimination during mating. The female discrimination behavior of both species has been mapped to separate loci on the second chromosome adjacent to the *Delta* locus (Doi et al., 2001). Populations of *D. melanogaster* from Zimbabwe (Z type) and populations from the rest of the world (M type) may be in the incipient stages of speciation due to sexual isolation caused by Z type females and M type males not mating (Hollocher et al., 1997; Wu et al., 1995). Z type populations have a mutation in the *desaturase2* gene, which correlates with a different female pheromone profile and the discriminatory mating behavior, but this gene is only one of seven loci identified as responsible for the changes in mating behavior between Z and M types (Fang, Takahashi, &

Wu, 2002; Takahashi et al., 2001; Ting, Takahashi, & Wu, 2001). The *period* gene has been shown to be involved in the isolation between *D. melanogaster* and *D. pseudoobscura*. The two species exhibit assortative mating as a result of being sexually active at different time ranges during the day, which correlates to different alleles of the *period* (Tauber & Eberl, 2003). The *period* gene also affects a species-specific aspect of the male courtship song. For example, female *D. melanogaster* and *D. simulans* demonstrate a preference for the courtship song of their own species, indicating that this behavior contributes to the isolation between these species (Kyriacou & Hall, 1982).

The *D. melanogaster Delta* gene is located on chromosome 3 and includes approximately 23,500 base pairs of DNA. There are three mRNAs transcribed from the *Delta* locus, both comprised of six exons, but differing in polyadenylation sites (Haenlin, Kramatschek, & Campos-Ortega, 1990). All three transcripts code for the same protein, which is a trans-membrane protein (Artavanis-Tsakonas, Matsuno, & Fortini, 1995; Haenlin, Kramatschek, & Campos-Ortega,1990). *Delta* belongs to the group of neurogenic genes in *Drosophila*, as many of its mutations result in extra nervous tissue and less epidermis. The main function of the *Delta* protein is to specify whether cells belong to the epithelial or neural lineage during larval development. It accomplishes this through cell signaling by interacting with Notch proteins on the surface of other cells. *Delta* on the surface of one cell binds to Notch on the surface of a neighboring cell, thereby activating Notch and allowing it to be cleaved inside the cell. Notch then moves into the nucleus, binding to another protein to form a transcription factor, which activates target genes coding for repressor proteins (Artavanis-Tsakonas, Matsuno, & Fortini, 1995). *Delta*'s mutations have phenotypes that affect the central nervous system as well as sensory organs, including the eyes and sensory

bristles (Haenlin, Kramatschek, & Campos-Ortega, 1990); thus, a change in *Delta* could affect the development of a behavior or change the perception of a stimulus, resulting in a different behavior. The *Delta* gene, as described earlier, has been implicated in the female discrimination behavior of *D. ananassae* and *D. pallidosa.* Because of these possibilities, *Delta* was chosen as a candidate gene for sequencing.

Detection of Mutations Associated with Behavior

Amplified fragment length polymorphisms (AFLPs) can detect differences in DNA sequences without having to sequence the DNA. Restriction enzymes cut the DNA into small pieces and adaptor sequences are ligated to the ends of these pieces. The adaptors contain sequences that are recognized by primers, which are used in the polymerase chain reaction (PCR) to amplify the pieces with adaptors. In a second round of PCR, more specific primers are used that amplify a more select number of the pieces (Vos et al., 1995). The DNA is then electrophoresed on an agarose gel to separate the pieces. A DNA band present in one sample, but not another, would indicate a sequence difference between the samples within a cut site of one of the restriction enzymes. The exact location of this difference within the genome is not known until the DNA band is cut out and sequenced. Any differences in the presence or absence of DNA fragments between selective lines that correspond to differences in the behavior of those lines would suggest the possibility that those DNA fragments contribute to the behavioral difference. AFLPs have largely been used in plant studies, but Luckinbill and Golenberg (2002) used AFLPs to map quantitative trait loci for longevity in *D. melanogaster* using artificially selected inbred lines (Bensch & Akesson, 2005).

The purpose of my research is to attempt to identify DNA elements that contribute to the genetic basis of female choice in *vg* mutants. In order to do this, I created several lines of *vg* mutants by selecting for *vg* females that choose to mate with *vg* males over *wt* males. I compared the DNA of the selection lines to the DNA of the original *vg* population and a *wt* population using AFLPs.

Specifically, my research has focused on the following:

1. Generation of RI lines that differ with regards to a specific behavior
2. AFLP analysis to assess genetic differences corresponding to behavioral differences
3. An examination of the *Delta* gene and its relation to behavioral differences

The results of this project could have significant implications for evolutionary biology and behavior genetics. It is possible that these two stocks of flies are in the incipient stages of speciation. Knowing the genes that cause behavioral isolation between these stocks could help to create a model of how sexual selection can drive evolution through genes governing sexual behaviors. Since very little is known about the genetic mechanisms of behavior, the identification of genes in model organisms, such as *Drosophila*, could potentially enhance the discovery of genes in other animals. The activity of a gene that influences a behavior could be traced through development to learn more about how behaviors are set up by genes. The success of this study could also exhibit the usefulness of using AFLPs in research on animals.

MATERIALS AND METHODS

Drosophila Stocks

Vestigial-winged (*vg*) and Oregon-R wild-type (*wt*) *Drosophila melanogaster* stocks were obtained from Carolina Biological Supply Company (Burlington, NC). Flies were raised on Ward's Blue Instant *Drosophila* Medium from Ward's Scientific Supply Company (Rochester, NY) and were kept at room temperature (approximately 25°C) with normal light conditions. All flies used in experiments were between 3 and 12 days old. Virgin females were collected within 6 hours of eclosion. Flies were anesthetized with CO_2 gas. To reduce the influence of exposure of CO_2 gas on behavior (Barron, 2000), individuals used in behavioral assays were allowed to recover a minimum of 24 hours after anesthetization.

Behavior Tests

The courtship behaviors of the stocks were tested to ensure that the previously reported behaviors (Aldridge, 2005; Stockdale, 2004) were still being observed. Males and virgin females of both *wt* and *vg* stocks were randomly mated to test the male's initial approach. One male and one female were placed in a clear plastic Petri dish and videotaped using a Canon ES80 8mm video camcorder on Hi-8 tape for approximately 30 minutes. Then the video was recorded onto VHS tapes for viewing. The initial angle at which the male approached the female during courtship was then scored from the video.

Virgin *vg* females were given a choice test to determine their preference for *vg* males versus *wt* males. Three to six virgin *vg* females were placed in a vial with an equal number of *vg* and *wt* males and allowed to mate for six hours. After six hours, the females were separated from the males and each was housed in a separate vial. Mate choice was assessed based on the phenotypes of each female's progeny. Because *vg* is a recessive mutation, if a *vg* female mates with a *wt* male, there will be no *vg* offspring. Therefore, if there are *vg* offspring, then the female mated with a *vg* male.

Recombinant Inbred Lines Through Artificial Selection

Recombinant inbred (RI) lines were created by following the selection regime outlined in Cochrane et al. (1998) and Aldridge (2005). Briefly, in each generation, virgin *vg* females were given a choice test as explained above and females that mated with *vg* males were selected for the next round. Selection was continued for ten generations and then the lines were brother-sister pair mated for an additional ten generations. After the first round of selection, the *vg* males used were the siblings of the *vg* female used. Thus, if the *vg* female chose a *vg* male, inbreeding would have occurred and this procedure should, after 15-20 generations, produce a stock of flies that are all >99% homozygous, but for different parts of the original *vg* genome (Cochrane et al., 1998). After ten generations of single-pair matings, the females from each RI line were tested again for their mate preference. Sixty flies from each line and from the *vg* and *wt* stocks were placed in 1.5ml microcentrifuge tubes and frozen at -20°C for molecular analysis.

Genomic DNA Extraction

Genomic DNA was isolated from the frozen flies using a DNeasy Blood and Tissue Kit from Qiagen (Valencia, CA). Sixty flies (approximately 50 mg) of each type were homogenized with PBS in a 1.5 ml microcentrifuge tube. The PBS was added in 3 increments of 60 μl each and the samples homogenized after each addition. Proteinase K and Buffer AL were then mixed in to each sample and the samples were incubated at 56°C for 10 minutes. As a modification to the protocol, 4 μl RNase A was added to each sample and the samples were incubated at room temperature for 2 minutes. Each mixture was then transferred by pipette into a DNeasy Mini spin column and centrifuged for 1 minute at 6,000 x g. The flow through was discarded and the samples were washed with Buffer AW1 and centrifuged again at the same speed for 1 minute. The flow through was again discarded and the samples were washed with Buffer AW2 and centrifuged at 20,000 x g for 3 minutes. Buffer AE was then applied by pipette to the membrane in each spin column over new collection tubes and the samples were centrifuged for 1 minute at 6,000 x g.

After extraction, a NanoDrop spectrophotometer (Nanodrop Technologies, Oxfordshire, U.K.) was used to measure the DNA concentration, 260/280 ratio, and 260/230 ratio of each sample. The DNA was then precipitated out of solution and re-suspended, as detailed below, to increase the concentration. To precipitate the DNA, 0.1 X volume of 3M sodium acetate and 2.5 X volume of 100% ethanol were added to each sample and the samples were frozen at -80°C for 1 hour. To pellet the DNA, each sample was then centrifuged for 15 minutes at maximum speed. The supernatant was poured out carefully so as not to disturb the pelleted DNA and then the DNA was washed with 70% ethanol and centrifuged for 5 minutes at maximum speed. The supernatant was again carefully poured off and the DNA was allowed

to air dry under a fume hood until all the residual ethanol had evaporated. Depending on the original DNA concentration, 25-50 μl TE was added to a sample to resuspend the DNA (more TE was added to samples with higher original concentrations to reduce the variability in DNA concentrations across samples).

Amplified Fragment Length Polymorphisms

AFLP analysis was conducted on all DNA samples. All AFLP reactions were performed using the AFLP Analysis System I kit from Invitrogen (Carlsbad, CA). There are four main reactions needed to perform AFLP analysis: restriction digest of genomic DNA, ligation of adaptors, preamplification reactions, and selective amplification. For the restriction digest, 5X reaction buffer, 250 ng DNA and *Eco*RI/*Mse*I were mixed together in 0.2 ml microcentrifuge tubes and briefly centrifuged. The samples were incubated at 37°C for 2 hours and then at 70°C for 15 minutes using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA). To ligate adaptors to the digested DNA, the adaptor ligation solution and T4 DNA ligase were added to each sample and the samples were incubated for 2 hours at room temperature (approximately 21°C). The ligation mixture was diluted by adding 90 μl TE buffer to 10μl of the reaction mixture for use in the next reaction. The results of the digestion were separated by electrophoresis in a 1% agarose gel for 1 hour at 100V to check for the presence of DNA.

For the preamplification reaction, digested DNA with ligated adaptors was combined with pre-amp primers, 10X PCR buffer plus Mg and *Taq* DNA polymerase. In the GeneAmp PCR system 9700 thermal cycler, the reactions incubated at 94°C for 30 seconds, at 56°C for 60 seconds, and at 72°C for 60 seconds for 20 cycles, ending in a soak at 4°C. The reaction

mixture was then diluted by mixing 3μl of the reaction mixture with 147 μl TE buffer. The results of the preamplification reaction were separated by electrophoresis in a 1% agarose gel for 1 hour at 100V to check for the presence of DNA.

Selective amplification uses one primer that recognizes the adaptor ligated to *Eco*RI cut sites plus 3 additional nucleotide bases and one primer that recognizes the adaptor ligated to *Mse*I cut sites plus 3 additional nucleotide bases. There are 8 different primers of each type for a total of 64 possible primer pair combinations. Primer pairs that were used were E-ACC with M-CAT, E-AGG with M-CAC, and E-ACT with M-CAG. For each sample, the particular *Eco*RI primer and *Mse*I primer were mixed with dNTPs, 10X PCR buffer plus MG, *Taq* DNA polymerase, and the diluted template DNA. These samples were then incubated in a thermal cycler for one cycle of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 60 seconds, then 12 cycles during which the annealing temperature was lowered 0.7°C each cycle, ending with 23 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds.

When this protocol produced no results, some troubleshooting was necessary. Luckinbill & Golenberg (2002) used the same kit on *D. melanogaster* DNA and found that they needed 4x the amount of *Taq* DNA polymerase, so that was tried but did not work. Next, a PCR cycle without a touchdown phase and extra annealing time was performed, specifically 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 90 seconds for 25 cycles with a final soak for 10 minutes at 72°C. This new PCR cycle did not produce any measurable product, so an increased concentration of DNA was used in the reaction. To achieve the increased concentration, the DNA was not diluted after the preamplification reactions. The approach was successful.

The products of the selective amplification were visualized on 3% agarose gels alongside Lambda DNA digested with *Eco*RI and *Hin*dIII as a size marker. They were separated by electrophoresis for 2 hours at 60 V. The DNA was stained by soaking the gels in a 0.5 μg/ml solution of ethidium bromide in the dark for approximately 15 minutes, and then rinsing them in a water bath. The gels were then transilluminated with UV light and photographed using an AlphaImager (Alpha Innotech, San Leandro, CA).

When different samples showed different bands of DNA on the gel, those bands were extracted to further analyze. To extract the DNA bands, a Montage Gel Extraction kit (Millipore, Billerica, MA) was used. The DNA bands were cut out of 1% agarose gels cast with 1X modified TAE buffer. Gels were placed on a transilluminator and bands of interest were cut out and placed into Gel Nebulizer vials. The vials were centrifuged for 10 minutes at 5,000 x g.

Samples were measured for DNA concentration, 260/280 ratio, and 260/230 ratio using a NanoDrop spectrophotometer. If the DNA concentration was low, identical samples were combined then precipitated and resuspended as described above, except that modified TAE buffer was used instead of TE buffer. The extracted DNA was sent to Cornell Biotechnology Resource Center (Ithaca, NY) for sequencing.

Sequencing the *Delta* Gene

Six pairs of PCR primers were designed to amplify the exons of the *D. melanogaster Delta* gene using the program ExonPrimer (Institute of Human Genetics, Munich, Germany) with reference sequences of *Delta* cDNA and genomic DNA from FlyBase.org as input. The primers were designed with the following criteria to ensure specificity and avoid self-

annealing: primer length 18-22 bases, T_m 55°C- 80°C, GC content 50-60%, T_m difference between primer pairs ≤ 4°C, fewest possible dimers, no stem-loop structures possible unless stem ≤3 base pairs.

Six of the DNA samples representative of lines with different behaviors were used in PCR reactions with the designed primers to separately amplify each of the *Delta* exons in each sample. Each PCR solution contained 0.125 µl *Taq* DNA polymerase, 2 µl DNA, 1 µl of each primer at a concentration of 20µM, 4.5 µl of a buffer and dNTP mixture, and distilled water to bring it to 25µl. The following temperatures were used for the PCR reactions: 5 minute hold at 95°C, [1 minute at 95°C (melting), 1 minute at 60°C (annealing), 1.5 minutes at 72°C (extension)] for 30 cycles, 10 minute hold at 72°C, hold at 4°C until taken out (Windelspecht, 1996; J. Lloyd-Cowden, personal communication). PCR products were visualized by electrophoresis on 1% agarose gels alongside Lambda DNA digested with *Eco*RI and *Hin*dIII as a size marker to check for the correct band size. DNA in the gels was stained and imaged as described above. If the correct band was found on a gel, the PCR product was purified using a QIAquick PCR Purification Kit following the manufacturer's protocol (Qiagen, Valencia, CA). The DNA was eluted using 30 µl purified water.

The samples were measured for DNA concentration and purity using the NanoDrop spectrophotometer and then sent to Cornell Biotechnology Resource Center for sequencing. Sequences that ran well were used in a nucleotide BLAST search optimized for highly similar sequences against the Nucleotide collection database to ensure the correct gene was amplified (Zhang, Schwartz, Wagner, & Miller, 2000). The ambiguous reads in each sequence were edited based on trace files using FinchTV (Geospiza, Seattle, WA).

The sequences were then aligned in contigs and analyzed for differences using CLC DNA Workbench (CLC Bio, Cambridge, MA). To focus on possible differences in coding regions of the *Delta* gene, one overall contig was created using the forward and backward reads from each of the six DNA samples for all six exons, with *Delta* cDNA as a reference. One contig per exon using *Delta* region genomic DNA as a reference was also created to examine the sequences beyond the intron/exon borders. Conflicts in the contigs were resolved wherever possible by examining and comparing the trace files of each sequence involved.

RESULTS

Behavior Tests

The behavior tests showed similar results as were previously indicated by Aldridge (2005) and Stockdale (2004). All *wt* males approached females from behind, while all *vg* males approached females from the side. *vg* females mated with *vg* males over *wt* males 43% (12/28) of the time.

Recombinant Inbred (RI) Lines

Twelve RI lines were created that ranged in *vg* preference behavior from 30% to 100% (Table 1). Mortality was high among RI lines due to bacterial growth and poor viability, as only 12 of 107 *vg* lines survived the entire experiment. No RI lines differed greatly in behavior from the last round of selection to the final behavior test after pair matings.

Table 1. Behavior of *vg* females in each RI line.

RI Line	% *vg* females mating with *vg* male over *wt* male	
	After selection	After pair-mating
Line A	80	80
Line B	25	30
Line C	100	100
Line D	60	60
Line E	100	100
Line F	50	50
Line G	43	40
Line H	100	100
Line I	70	67
Line J	75	80
Line K	100	100
Line L	80	80

Genomic DNA Extraction

DNA was successfully extracted from all RI lines. The DNA concentration and purity after precipitation and resuspension varied among all samples, but the concentrations and purities were high enough in all samples to use in further molecular analysis (Table 2).

Table 2. Concentration and purity of extracted DNA. 260/280 is the ratio of absorbance of the DNA sample at 260nm and 280nm and 260/230 is the ratio of absorbance of the DNA sample at 260nm and 230nm.

Sample	Concentration (ng/μl)	260/280	260/230
Line A	256.7	1.19	0.53
Line B	285.8	2.08	1.31
Line C	260.5	2.00	1.18
Line D	257.1	1.94	1.05
Line E	146.9	1.56	0.56
Line F	195.0	1.21	0.47
Line G	124.6	1.83	0.47
Line H	236.6	2.11	1.27
Line I	92.4	1.97	0.69
Line J	142.2	1.87	0.87
Line K	188.2	1.93	0.64
Line L	130.3	1.69	0.86
vg	131.2	1.66	0.73
wt	134.4	1.68	0.69

Amplified Fragment Length Polymorphisms (AFLP)

The AFLP results were inconclusive. Each DNA sample was visualized as a smear on a gel after the restriction digest and preamplification reactions. The three primer pairs used during selective amplification produced a different banding pattern for each DNA sample examined (Figure 1). Within each primer pair's reactions, most samples exhibited unique banding patterns. Many samples did not show distinguishable bands or showed a mixture of smears and bands. Distinguishable bands from the E-ACC/M-CAT reaction were gel extracted and measured for concentration and purity (Figure1b; Table 3). Despite efforts to increase DNA concentration, none of the extracted DNA bands were successfully sequenced. All of the sequencing reactions failed, most likely meaning that the sequencing reactions didn't happen because the primers failed to bind. Several of the sequence files did contain a short sequence (100-150 bp), but when used in a BLAST search, the sequences returned no results because there were too many ambiguous reads within the sequence.

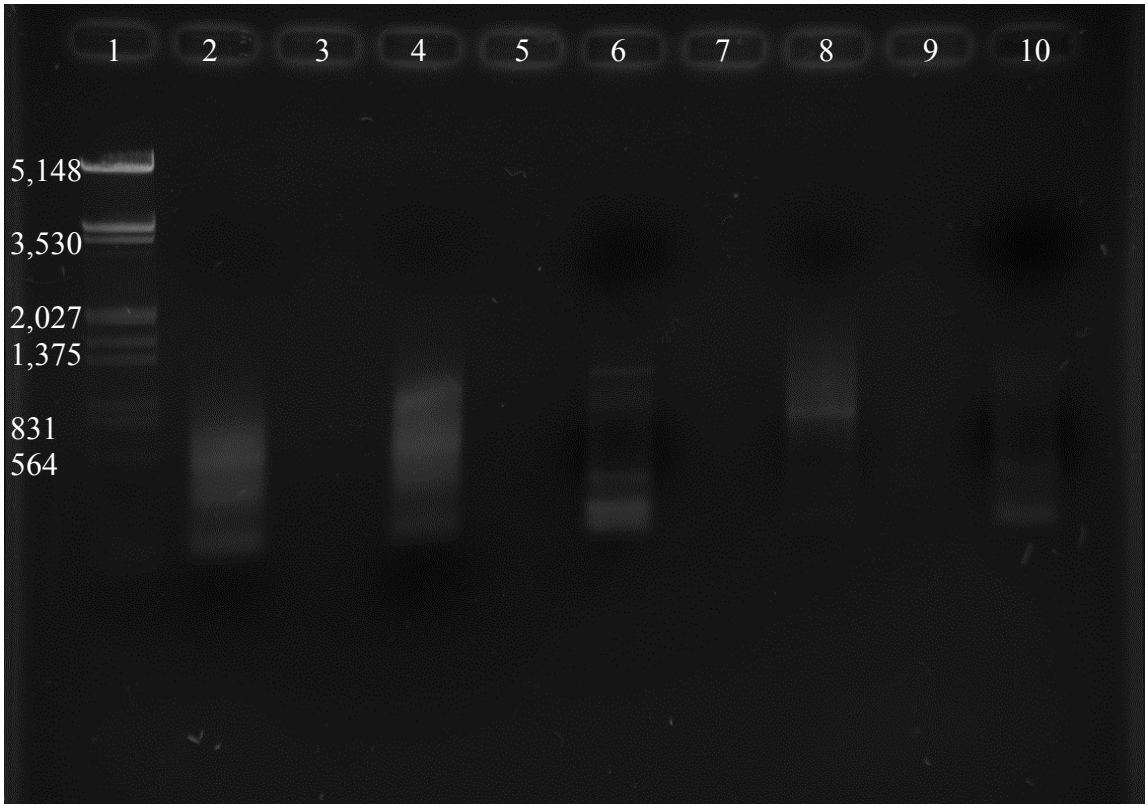

Figure 1a. AFLP Gel Electrophoresis results. PCR performed with M-CAC and E-AGG primers. The contents of each lane are as follows: lane 1, size marker; lane 2, Line B; lane 4, Line C; lane 6, Line F; lane 8, Line J; Lane 10, *vg* stock; all other lanes are empty. Sizes of marker fragments are indicated in base pairs.

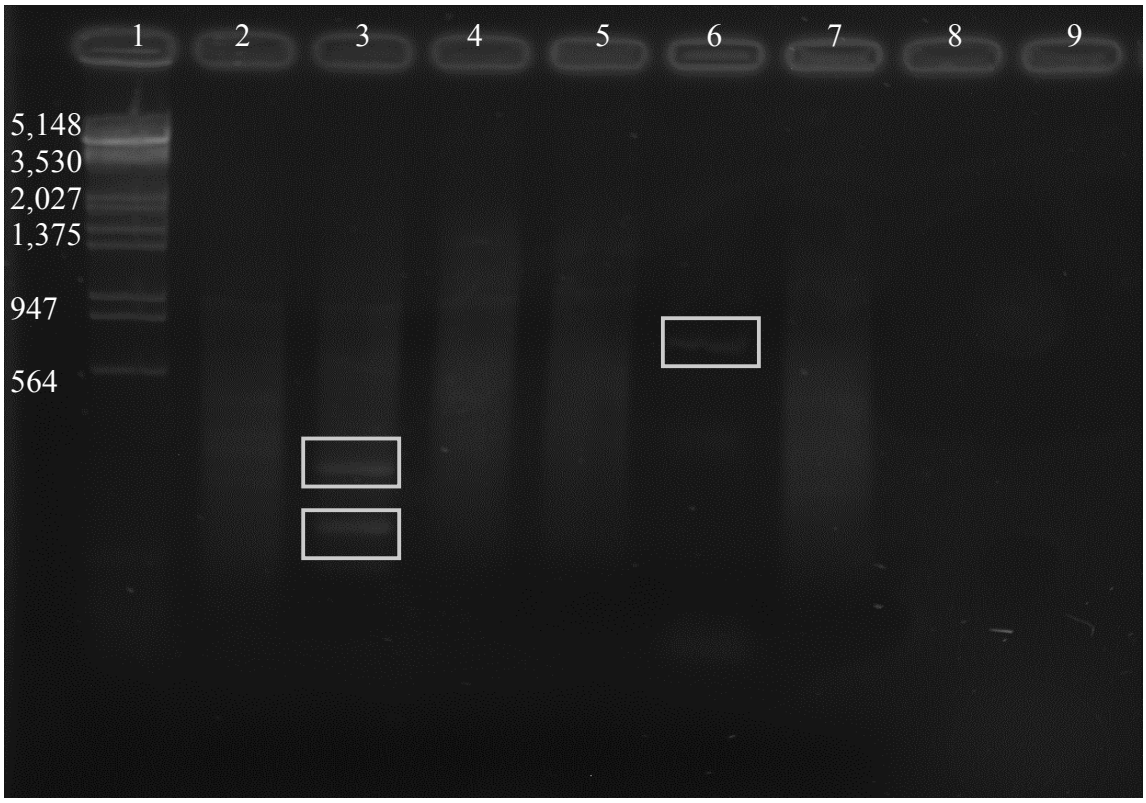

Figure 1b. AFLP Gel Electrophoresis results. PCR performed with M-CAT and E-ACC primers. The contents of each lane are as follows: lane 1, size marker; lane 2, Line A; lane 3, Line D; lane 4, Line E; lane 5, Line G; lane 6, *vg* stock; lane 7, *wt* stock; all other lanes are empty. The boxes indicate which bands were gel extracted. Sizes of marker fragments are indicated in base pairs.

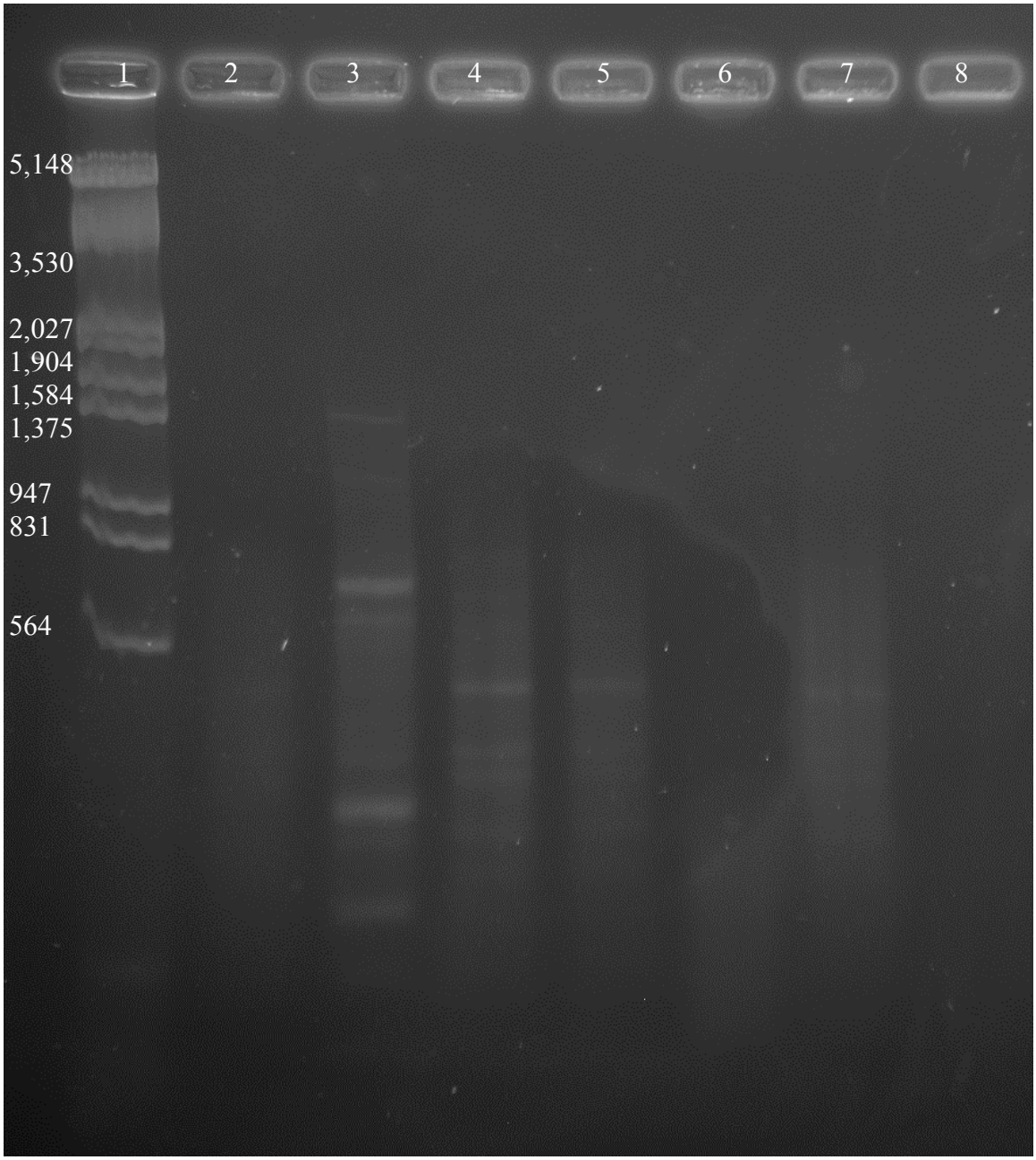

Figure 1c. AFLP Gel Electrophoresis results. PCR performed with M-CAG and E-ACT primers. The contents of each lane are as follows: lane 1, size marker; lane 2, Line A; lane 3, Line D; lane 4, Line E; lane 5, Line G; lane 6, *vg* stock; lane 7, *wt* stock; all other lanes are empty. Sizes of marker fragments are indicated in base pairs.

Table 3. Gel extraction results. A result of "failed" indicates that the sequencing reaction produced no results.

Experiment	Band	Concentration (ng/μl)	260/280	260/230	Sequencing Result
Original	E1	2.6	1.09	0.14	failed
	E2	9.0	1.64	0.36	failed
	J	4.7	1.05	0.22	failed
More DNA in selective PCR	E1	10.3	1.22	0.80	failed
	E2	14.5	1.71	0.92	failed
	J	21.2	1.70	0.84	failed
More DNA into preamp reactions	E1	29.1	1.69	1.01	failed
	E2	37.0	1.65	1.14	failed
	D	8.6	1.59	0.67	failed
	J	33.3	1.73	1.09	failed
Combine bands, precipitate, resuspend	E1	<1	-0.01	0.00	failed
	E2	8.0	1.55	0.62	failed
	E3	6.4	1.51	1.36	failed
	J	32.9	1.51	0.60	failed

Sequencing the *Delta* Gene

Six PCR primer pairs were designed, each with similar properties but different target sequences (Table 3). All of the primer pairs amplified the correct sequence using an annealing temperature of 60°C. All DNA samples used produced the correct band size for each of the primer pairs, except for the wild type DNA, which produced no bands and had to be redone (Figure 2, Figure 3). DNA concentrations after PCR purification varied greatly among the samples, but all samples produced sequencing results (Table 4). When used in a BLAST search, all sequences returned the expected result, *D. melanogaster* chromosome 3R (accession number: AE014297.2) as the first hit and *D. melanogaster Delta* protein gene (accession number: BK004004.1) as the second hit, with E values much less than 1 (Table 4; Zhang et al., 2000). All ambiguous reads in the sequence files were edited where possible based on the trace files (Figure 4).

Table 4. Designed primers. Primers made to sequence the coding regions of the *Delta* gene and their properties. Tm is the melting temperature in degrees Celsius. Target length and Product size are in units of base pairs.

Primer Sequence	Tm	%GC	Target	Target length	Product Size
CACAGCCCGTGGATTATTAC	60.4	50	Exon 1	57	217
GCGTGCGCGTAGAAATAAAG	60.4	50	Exon 1		
GGGAGCTGCATTCTTTGTTC	60.4	50	Exon 2	312	635
TGACAGCGAAAAGTGTGTGG	60.4	50	Exon 2		
GCCTGTCAATTTCCCGTTTAG	60.4	50	Exon 3	67	211
TTGCCCATGTTTCAAGGAAG	58.4	45	Exon 3		
TTCATTAGTCTATCGCCGGG	60.4	50	Exon 4	249	392
TTTAAGACAACCCCTCCCAC	60.4	50	Exon 4		
GTGGGGAGTCCATTTCAAAG	60.4	50	Exon 5	55	371
AACCCGTTAGTTGCAGTTGG	60.4	50	Exon 5		
ATTCACATAAAGGCATGGGC	58.4	45	Exon 6	440	590
CTGTAGCCAATGGGACATTC	60.4	50	Exon 6		

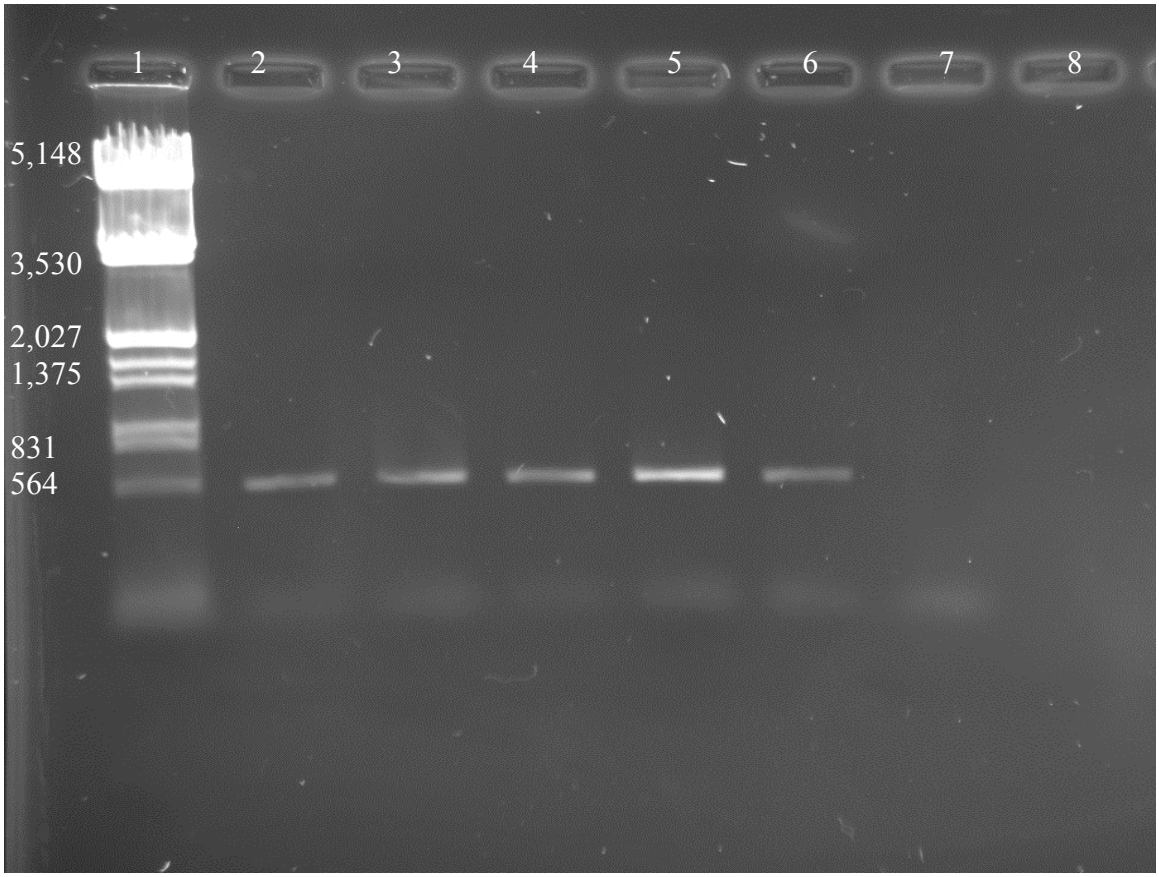

Figure 2. *Delta* exon 6 gel electrophoresis results. Lanes 1-7 contain the following: size marker, PCR products from Line A, Line B, Line C, Line D, *vg* stock, *wt* stock. Sizes of marker fragments are indicated in base pairs.

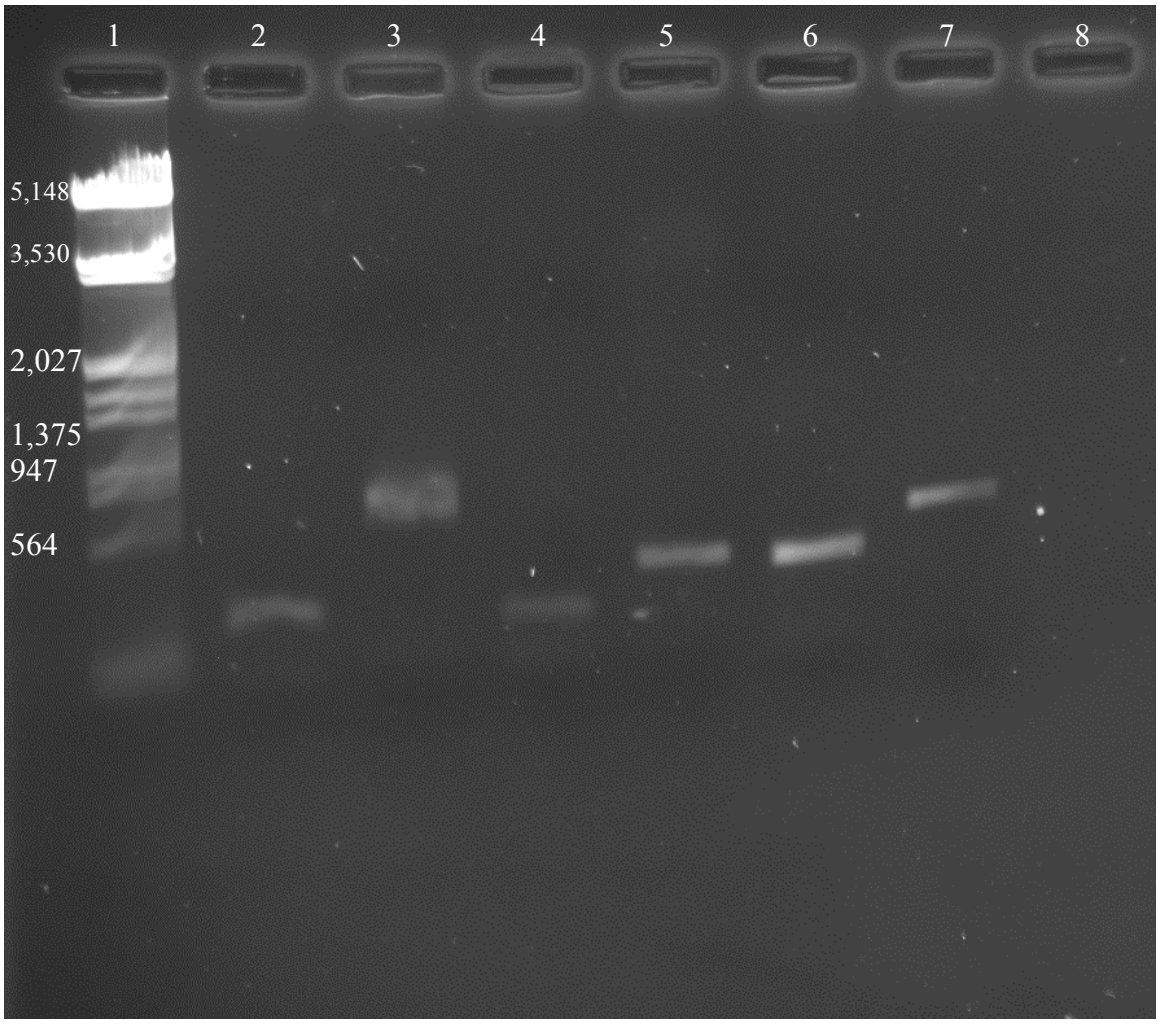

Figure 3. Wild type DNA Gel electrophoresis results. PCR performed with each of the six *Delta* primer pairs. Lane 1 contains size marker. Lanes 2-7 contain *wt* DNA with: exon 1 primers, exon 2 primers, exon 3 primers, exon 4 primers, exon 5 primers, exon 6 primers. Sizes of marker fragments are indicated in base pairs.

Table 5. *Delta* sequencing results. 260/280 is the ratio of absorbance of the DNA sample at 260nm and 280nm and 260/230 is the ratio of absorbance of the DNA sample at 260nm and 230nm. "Overlap" in the sequencing results indicates that there was interference in the sequencing read resulting in multiple ambiguous reads. E-value is for the first result returned in a BLAST search, which was always *D. melanogaster* chromosome 3R.

Primer Pair	DNA Sample	Concentration (ng/μl)	260/280	260/230	Sequence Result	BLAST E-value
exon 1	a	69.4	1.78	1.04	Successful	2×10^{-79}
	b	60.5	1.84	1.09	Successful	2×10^{-83}
	c	49.6	1.82	1.08	Successful	3×10^{-81}
	d	54.9	1.85	1.01	Successful	4×10^{-80}
	vg	41.3	1.78	0.75	Successful	2×10^{-79}
	wt	45.1	1.78	1.12	Successful	2×10^{-83}
exon 2	a	66.3	1.81	1.23	Successful	0.0
	b	168.1	1.57	1.19	Successful	0.0
	c	157.5	1.58	1.14	Successful	0.0
	d	59.2	1.74	1.00	Overlap on forward read	0.0
	vg	159.4	1.43	0.99	Successful	0.0
	wt	50.2	1.84	0.96	Successful	0.0
exon 3	a	47.7	1.63	0.68	Successful	0.0
	b	35.3	1.81	1.03	Successful	1×10^{-75}
	c	70.1	1.64	0.86	Successful	1×10^{-75}
	d	24.2	1.87	1.56	Successful	2×10^{-73}
	vg	26.6	1.71	0.81	Successful	4×10^{-75}
	wt	44.6	1.77	1.07	Successful	2×10^{-74}
exon 4	a	25.3	1.69	0.81	Successful	3×10^{-179}
	v	46.0	1.67	0.90	Successful	6×10^{-176}
	c	52.0	1.79	0.96	Successful	6×10^{-176}
	d	53.1	1.78	0.91	Successful	6×10^{-176}
	vg	50.5	1.62	0.77	Successful	6×10^{-176}
	wt	44.6	1.82	1.14	Successful	6×10^{-176}
exon 5	a	53.7	1.68	0.83	Successful	8×10^{-160}
	b	69.0	1.68	0.96	Successful	1×10^{-157}
	c	58.2	1.78	1.17	Successful	1×10^{-157}
	d	52.1	1.71	0.93	Successful	1×10^{-157}
	vg	63.6	1.75	1.04	Successful	1×10^{-157}
	wt	40.4	1.79	1.30	Successful	1×10^{-157}
exon 6	a	37.0	1.72	1.05	Successful	0.0
	b	41.7	1.76	1.00	Overlap on reverse read	5×10^{-164}
	c	55.5	2.22	1.02	Successful	0.0
	d	36.9	1.93	1.62	Successful	0.0
	vg	33.8	1.73	0.90	Successful	0.0
	wt	50.5	1.82	1.25	Successful	0.0

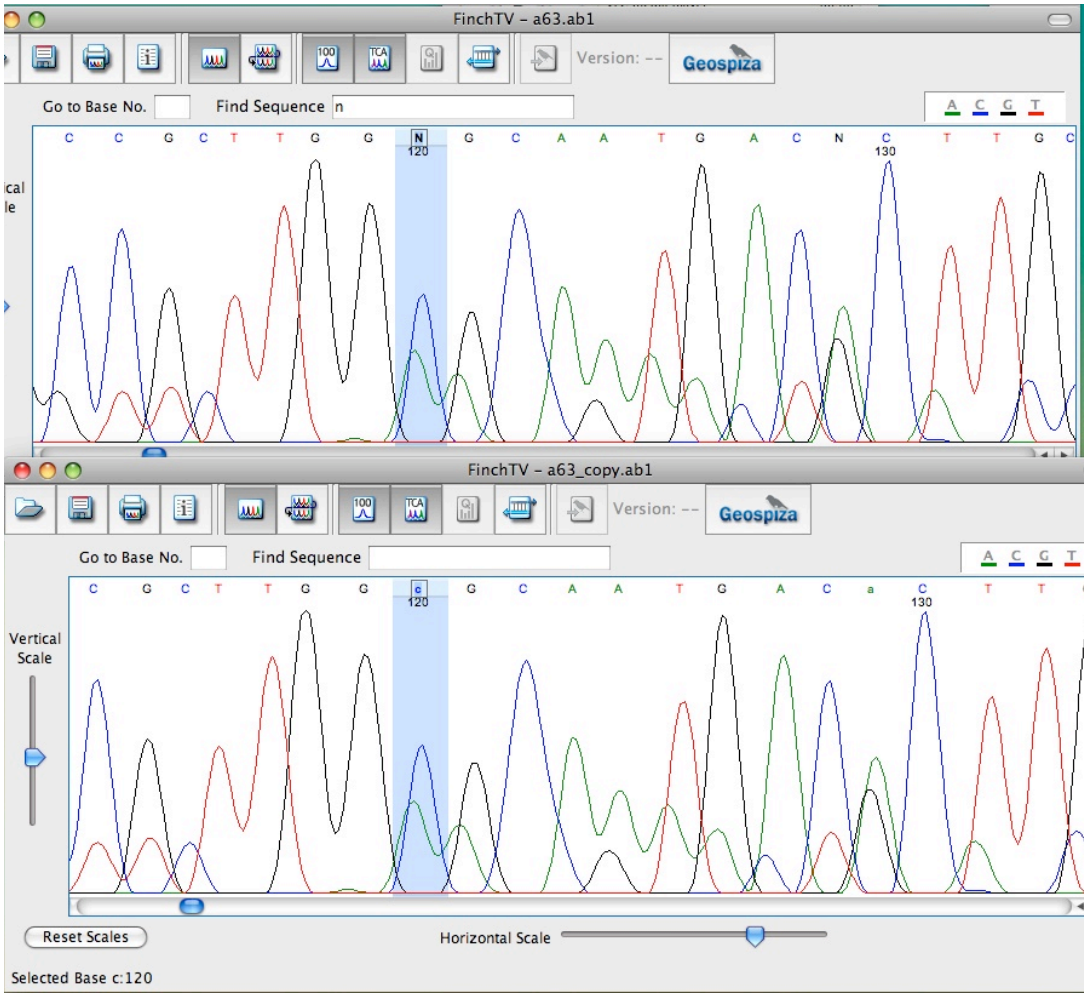

Figure 4. Screenshot sample of sequence editing. The top trace file is an unedited sequence, the bottom trace file is the same sequence after editing. The highlighted base has been changed from 'N' to 'c'.

Contig analysis revealed no significant differences among sequences. Overall, there were 178 conflicts in the individual exon contigs and 93 conflicts in the cDNA contig, but only 25 of the former and 6 of the latter were unresolved after reviewing the trace data (Figure 5). Of the unresolved conflicts, 14 were instances where all of the experimental data agreed but were different from the reference sequence. There were two cases in the exon 2 contig where the genomic reference sequence had 6 and 2 extra bases within a repetitive sequence. All the rest of the sequence conflicts were the result of the forward and reverse

reads disagreeing. There were no instances where the forward and reverse reads from one DNA sample matched and were different from the other sequences.

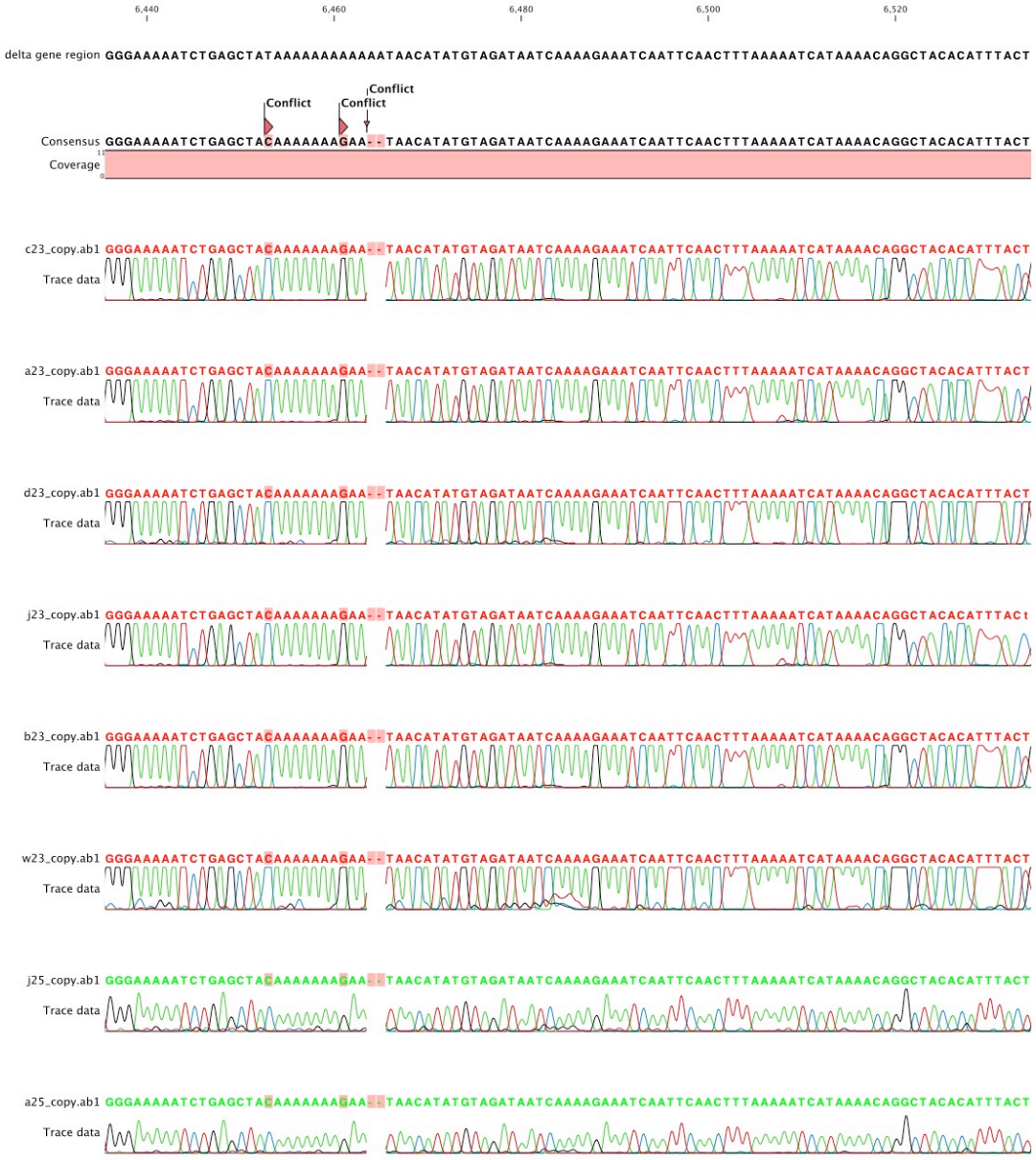

Figure 5. Excerpt of *Delta* exon 2 contig. *Delta* gene region sequence used as reference.

DISCUSSION

The purpose of this study was to identify genetic elements that are linked to a behavior exhibited in *D. melanogaster vg* mutants through the use of RI lines and molecular analysis. The AFLP procedure was originally chosen to screen the genomes of RI lines of flies for genetic differences that correspond to their differences in behavior. The differences illuminated by the AFLPs were to be identified through sequencing. Because the sequencing failed, a new approach was taken, which involved the analysis of a single candidate gene.

All *vg* males tested showed a side approach to the female while all *wt* males showed a rear approach. These results are identical to the results found by Stockdale (2004) and J. Lloyd-Cowden (personal communication). Because all of the males within a group showed the same behavior and this totality has not changed over time, it is likely that the alleles for male approach behavior are fixed in both the Oregon-R *wt* and the *vg* populations. There is always the chance that had the sample sizes in these experiments been much larger, a small percentage of flies that differed in their behavior from their group may have been found. Because of the fixation of the alleles in both populations, it would make sense that the allele for rear approach was originally fixed in the Oregon-R *wt* population. A mutation causing the side approach appeared in the *vg* population and must have been beneficial, perhaps in some way making up for the lack of wing vibrations during courtship, causing the mutation to be passed on more and more often than the rear-approach allele until it was fixed in the

vg population. Alternatively, the simplest explanation would be that the *vg* mutation itself also causes the difference in behavior. This possibility seems unlikely as the *vg* gene and its mutations have been extensively researched and have only been found to affect wing development.

Forty-three percent of *vg* females in my study mated with *vg* males over *wt* males. This figure is somewhat less than the 59% of *vg* females that mated with *vg* males in the study by Aldridge (2005). The percentage has changed over time and is not close to either extreme (0% or 100%), suggesting that alleles for female mate choice are fluctuating, rather than fixed, in the *vg* population. Aldridge (2005) also found that *wt* females chose *vg* males over *wt* males 51% of the time, indicating that this allele is not fixed in the Oregon-R *wt* population either. Because these populations are lab strains and are physically isolated from one another, a gene for preference of one type over another is a moot point and would be neutral in terms of evolutionary fitness. Thus, it is understandable that the female behaviors, and therefore the underlying alleles, fluctuate over time. Only in a situation where the two populations of flies were able to interact could a mating preference possibly have an evolutionary advantage or disadvantage.

My approach to RI lines was somewhat modified from the norm. The typical procedure for producing RI lines begins with crossing 2 progenitor lines that differ completely in the trait of interest (Bailey, 1971; Taylor, 1976). The *vg* females are a mixed population with regards to the trait of interest, so the procedure was modified to reflect this fact. Since two progenitor lines distinct in their behavior did not exist, the *vg* population was treated as if it were the result of crossing two progenitor lines and that initial step was skipped. Then, instead of completely random brother-sister pair matings, there was selection

for a trait. These procedural changes should not have affected the estimation that after 20 generations of inbreeding, approximately 99% homozygosity would be reached in all lines (Dixon, 1993).

Despite high mortality (89% of lines started failed), a sufficient number of RI lines were produced to allow their use in further analysis. This level of mortality is similar to that found in previous studies using *Drosophila* in RI lines; Aldridge (2005) lost about 70% of all RI lines by the sixth generation, while Cochrane et al. (1998) lost 82.4% of lines started. The high amount of homozygosity resulting in inbreeding depression accounts for most of the mortality, although bacterial growth on the food was also a culprit. Because of intense inbreeding depression, there is an inherent, unavoidable amount of selection for viability in the RI line procedure. It's only possible to use those individuals that have survived to start a new generation. Because of this selection, it is possible that in my own later analysis, when comparing the RI lines to the *vg* or *wt* population, that any differences may not be the result of different behaviors. It should not create a problem when comparing among the RI lines because they would have all been subjected to roughly the same amount of selection for viability.

Even though the lines were subjected to selection for increased mating with *vg* males, there were some lines that decreased in percentage of females mating with *vg* males. This decrease may have been the result of alleles combining in new ways to produce the opposite of the expected effect. Genes that are linked have a greater chance of recombination in RI lines as compared to regular crossing because the RI lines have gone through many more rounds of meiosis before reaching homozygosity. Because of this increased recombination, combinations of alleles that may not have existed or were rare in the original population are

expected. In any case, the RI lines displayed a range of behaviors, which was beneficial for later comparisons.

The AFLP experiment was, overall, inconclusive in that it was unable to provide any potential genetic elements correlated to the *vg* female behavior. Though it was clear that the AFLP procedure itself was successful, I was unable to garner any useful information from it in regards to my research question. There were no apparent correlations between the behavior of the different groups and their banding patterns. In other words, there was never a case where two groups with the same or similar behavior showed the same banding patterns while other groups showed different banding patterns.

In the M-CAG and E-ACT primer pair reaction, the RI lines with 40% and 100% *vg* choice and the wild-type show identical bands on the gel, while the RI line with 60% *vg* choice has a very different pattern (Figure 1c). The RI line with 60% *vg* choice and the *vg* stock both show only smears. In this case, the RI lines at either end of the spectrum and the *wt*, whose behavior is in the middle, had the same result, so the AFLP results do not correlate with the different behaviors. In the M-CAC and E-AGG primer pair reaction, the 30% and 100% choice lines show very similar smear patterns, the 50% choice line and the *vg* stock show similar, but not identical, smear patterns, and the 80% choice line shows a pattern different from all others (Figure 1a). Although the 50% choice line and the *vg* stock have comparable behaviors and results that are alike, the analogous results of the lines with different behaviors keep there from being a correlation between AFLP results and behavior in this case. In the M-CAT and E-ACC primer pair reaction, there were not many discernable bands, but the overall pattern of the 80% and 60% choice lines is quite different, while the overall pattern of the 100% and 40% choice lines is very similar (Figure 1b). Again, the

similar results come from lines with different behaviors. My initial plan was to go through more primer combinations and a correlation between the results and the behaviors may have shown up in a combination I did not try, but I didn't continue with this research after the subsequent part failed for the first set of primers.

Initially, I did not expect there to be so many differences in the AFLP results for each RI line. Assuming that the genetic cause of the difference in female behavior is a small mutation, as there is no other discernable phenotypic difference, and that all of the RI lines were derived from the *vg* stock, which is, itself, being a laboratory stock, already inbred, I expected that the RI lines would largely have the same results with a handful of bands differing between them. In performing the same selective amplification multiple times, I noticed that identical results were produced when using products of the same preamplification reaction, but somewhat different results were produced when using products of a different preamplification reaction. As seen in the gel extraction results, certain bands were always produced (E1, E2, and J) regardless of the preamplification reaction product used, while others (D and E3) were only seen when different preamplification reaction products were used (Table 3). Since any specific reaction might not showcase all of the bands that could be produced from a DNA sample with one pair of primers, it is difficult to determine whether all of the differences seen on the gels are in fact differences and not just bands that weren't produced this time.

Another potential failing of AFLP analysis would be that if the mutation causing the different behavior were small and weren't within the sequence recognized by the restriction enzymes used, it wouldn't show up as a different banding pattern. Only if a point mutation

were within the target sequence or if the mutation were a large insertion or deletion, would a group with the mutation show different sized bands.

The sequencing of gel-extracted bands was unsuccessful. Initially, it seemed that the concentrations of the DNA samples after extraction were not high enough, as they were all less than 10 ng/ml. After troubleshooting, several of the gel-extracted samples had concentrations equal to or greater than later PCR samples that were sequenced well; the highest concentration of a gel-extracted band was 37.0 ng/ml, while the lowest concentration of a PCR sample that sequenced well was 24.2 ng/ml. This problem could have been caused by different DNA pieces of the same size being in one band. The 3% agarose gel used may not have offered sufficient separation and several similar sized bands could have appeared as one band. Luckinbill and Golenberg (2002) used polyacrylamide gels and cloned the gel-extracted bands before sequencing in their similar study. While time and resources were too limited to use these methods in my experiment, my results indicated that these procedures would most likely be useful in future experiments.

Because the AFLP analysis did not deliver results, a candidate gene was chosen to be sequenced. Choosing a single candidate gene out of nearly 14,000 genes in the *D. melanogaster* genome is a difficult task. *Delta* was chosen as a candidate gene because of its implication in the behavioral isolation of *D. ananassae* and *D. pallidosa* and because of its involvement with the nervous system during development. Other genes that were considered included *dissatisfaction* and *spinster*, which influence female receptivity to mating (Finley, Taylor, Milstein, & McKeown, 1997; Suzuki, Juni, & Yamamoto, 1997) and *Period* and *cacophony*, which are within regions identified as being related to the male *vg* behavior (Stockdale, 2004).

Sequencing the *Delta* gene was successful, but ultimately unfruitful. Based on contig analysis, there were no differences in the *Delta* exon sequences among all lines tested but *Delta* cannot completely be ruled out as a candidate gene. I did not sequence the introns, which could contain regulatory sequences. It is still possible that *Delta* could be up or down-regulated in *vg* females because of a mutation in a regulatory sequence or the sequence of a gene that codes for a regulatory protein. This result was a small step toward understanding the exact genetic nature of this behavior difference, but at the pace of one gene at a time, it would take more than a lifetime to find an answer.

A genomic approach would be best suited to this type of research question. If resources and time were infinite, the best way to answer the question at hand would be to sequence the genomes of the RI lines, align them all, and analyze the differences. However, this approach was cost and time prohibitive. AFLP analysis should have been a much simpler way to get enough results to address my research question, but other types of genomic methods could also work in this situation. Restriction fragment length polymorphism analysis, a technique very similar to AFLP analysis, could work. Like AFLP analysis, RFLP analysis uses restriction enzymes to digest DNA, but RFLP analysis involves Southern blotting after gel electrophoresis to identify sequences of interest. This technique would encounter the same difficulties as sequencing a candidate gene; it would be troublesome to decide what sequence(s) to use as a probe in Southern blotting, without trying to map the entire genome.

Rather than target the differences in gene sequences among behavior groups, differences in transcription levels for the entire genome of each RI line could be detected by microarray as in Mackay et al. (2005). The results of microarray analysis could be used to

find differences in gene sequence since, in theory, any change in gene expression would be the result of a change in DNA sequence somewhere. However, using microarray analysis to find sequence differences is not as direct a path as could be hoped for, it would be quite a long process and it could be difficult to trace expression differences back to the sequence differences that caused them, especially if not much is known about the regulation of the genes that show in the results.

The purpose of this study was to test the efficacy of these types of methodological approaches to the question at hand. The first method, AFLPs, did not produce usable sequence results. While the second method, sequencing a candidate gene, did produce results, it would take many iterations of this method to fully answer the question or more information would be needed to pick candidate genes. The success of this study lies in its illumination of the downfalls of these methodologies.

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